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{Exhibit 42}

Sodja and Davidson, "Gene Mapping and Gene Enrichment by the Avidin-Biotin Interaction: Use of Cytochrome-C as a Polyamine Bridge," Nucl. Acids. Res., 5, pp. 385-400 (1978)

Gene mapping and gene enrichment by the avidin-biotin interaction: use of cytochrome-c as a polyamine bridge

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ABSTRACT

A modification of previously described methods of electron microscopic gene mapping and of gene enrichment based on the avidin-biotin interaction is presented. The modification consists of coupling cytochrome-c instead of pentane diamine to the oxidized 2', 3' terminus of an RNA by Schiff base formation and BH_4 reduction. The RNA-cytochrome-c conjugate is purified by a simple chromatographic procedure; several biotins are attached to the cytochrome moiety by acylation. The extended arm between biotin and RNA gives efficient electron microscopic gene mapping of DNA:RNA-biotin hybrids with avidin-ferritin and avidin-polymethacrylate sphere labels and efficient gene enrichment by buoyant banding of DNA:RNA-biotin:avidin-spheres in CsCl . A 1400 fold enrichment (thus, 25% pure) and a 90% yield of long *Drosophila* DNA strands with 5S RNA genes is achieved.

INTRODUCTION

The preceding paper (1) describes a method of mapping, with a ferritin label, a short RNA:DNA hybrid region along a single stranded segment of DNA. The essential features of the method are: a) covalent attachment of biotin to the periodate oxidized 3' terminus of the RNA by a diamine bridge, using a simple diamine such as $\text{NH}_2(\text{CH}_2)_5\text{NH}_2$; b) covalent attachment of avidin to the electron opaque label ferritin; c) hybridization of the covalent tRNA-biotin conjugate to a single stranded segment of DNA that contains the coding sequence (gene) for the tRNA; d) electron microscopic mapping of the position of the hybridized tRNA-biotin along the single strand segment of DNA after binding of ferritin-avidin to the biotin. This method gives a moderately satisfactory overall efficiency of gene labeling, and has been used to map the tRNA genes of HeLa mitochondrial DNA (2). We describe here an improved method in which we use a defined polyamine instead of a diamine as the bridge between the 3' end of the RNA and the carboxylic acid biotin. The polyamine used is the protein, cytochrome-c. This bridge is believed to have several advantages: a) it is probably more extended than the pentane diamine; b) several biotins can be attached to one RNA molecule; c) the

purification step for tRNA-biotin with the pentane diamine bridge involves elution from avidin sepharose at pH 2.5 in 6M guanidine hydrochloride. These fairly drastic conditions are replaced for purification of tRNA-cytochrome-c-biotin (tRNA-cc-biotin) by hydroxyapatite chromatography at neutral pH, thus diminishing the probability of chemical degradation of the labeled RNA. As a result, presumably, of items a, b, and/or c, the overall efficiency of gene labeling with ferritin in the electron microscope by the new method is an improvement over that achieved previously. d) with the new extended bridge but not with the diamine bridge, avidin attached to polymethylmethacrylate spheres (3) will label DNA:tRNA-biotin hybrids.

Furthermore, the binding of avidin-spheres by DNA:RNA-biotin molecules forms the basis for a gene enrichment procedure. The polymer spheres used have a density and molecular weight of about 1.25 g/ml and 7.6×10^7 daltons, respectively. The spheres can be used as relatively massive floats to separate DNA:RNA-biotin-avidin-sphere molecules from unhybridized DNA strands by buoyant banding in CsCl, thus accomplishing gene enrichment. Manning, Pellegrini and collaborators have demonstrated this application of the avidin-biotin labeling approach for long RNA molecules, by enriching for the rDNA genes of *Drosophila* and for the histone genes of the sea urchin (4,5). In these cases, it is appropriate and convenient to attach cytochrome-c-biotin nonspecifically to the RNA by CH₂O crosslinks. In the present communication, we show that 3' terminal labeling of 5S RNA by cytochrome-c can be used for highly efficient gene enrichment of the *Drosophila* 5S RNA genes.

The basic reaction scheme of the present procedure is:

- 1) Oxidation of free 2', 3' OH ends of RNA to the dialdehyde with periodate.
- 2) Schiff base formation of the terminal dialdehyde with the polyamine, cytochrome-c, at relatively low ionic strength, and stabilization of the compound against dissociation and/or β elimination by BH₄⁻ reduction.
- 3) Purification of RNA-cytochrome-c from free RNA and free cytochrome-c by sequential chromatography on carboxymethyl cellulose (CMC) and on hydroxyapatite (HAP).
- 4) Covalent attachment of several biotin molecules to lysine NH₂ groups of the cytochrome-c by acylation with the N-hydroxy succinimide (NHS) ester of the carboxylic acid biotin.
- 5) Hybridization of the RNA-cc-biotin to DNA.
- 6) Labeling with avidin-ferritin or avidin-spheres.

7) Gene mapping by electron microscopy or gene enrichment by banding in CsCl.

MATERIALS AND METHODS

Nucleic Acids. *E. coli* tRNA's, $\phi 80$ and $\phi 80$ psu_3^- DNA's, were obtained as described (1). *Drosophila* (Dm) 4S and 5S RNA and the *Drosophila* plasmids, pCIT19 and pCIT12, were prepared as previously described (6,7). Unlabeled as well as ^3H -labeled Dm DNA's were isolated from Schneider's line 2 tissue culture cells. Cells were labeled by the addition of 1.5 m Ci of ^3H -thymidine (Amersham Radiochemical Center, 41 Ci/mmol) to 50 ml of cells grown in suspension with gentle swirling. Cell density at the first addition of label was $1-2 \times 10^6$ ml; label was added in four equal fractions at 6 hr intervals, and cells grown for another generation (approximately 24 hrs) after the last addition. Cells were harvested by centrifugation at 2400 rpm at 0°C for 5 minutes, lysed by homogenization (10-15 strokes) in 0.5 M Tris base 0.025 M KCl, 5 mM $\text{Mg}(\text{Ac})_2$, 0.35 M sucrose, pH 7.6. DNA was prepared from this lysate by the procedure of Manning et al. (8). The specific activities of two separate preparations were 5.4 and 0.84×10^5 cpm/ μg .

Cytochrome-c. Commercial cytochrome-c (horse heart, type VI, Sigma) is contaminated with RNAase, which has approximately the same molecular weight and charge. RNAase was inactivated by treatment with iodoacetate by a modification of published procedures (9,10). Cytochrome-c (60 mg) was dissolved in 1 ml of 0.2 M NaAc buffer (pH 5.5). An equal weight of iodoacetate was added, the pH readjusted to 5.5 with concentrated NaOH, and the solution diluted to a final volume of 2 ml. The solution was incubated for 1 hr at 55°C and then dialyzed extensively at 0°C against 0.01 M sodium phosphate buffer (NaP), pH 6.8, and lastly against 0.1 M $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer, pH 9.2.

Preparation and Purification of RNA-Cytochrome-c. tRNA or 5S RNA were heated at 80° for 1-8 min in 1 mM NaAc buffer pH 6.8, cooled, adjusted to 0.1 M NaAc buffer (pH 4.8) and treated with periodate as previously described (1). The amount of RNA used was 0.5 - 1 mg in 0.5 - 1 ml of reaction mixture.

Oxidized RNA was dialyzed against 0.1 M $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer (pH 9.2) at 0°C . A 10-15 fold molar excess of cytochrome-c was added and the solution

ABBREVIATIONS

RNA-cc-biotin, 4S or 5S RNA-cytochrome-c-biotin; HAP, hydroxyapatite; CMC, carboxymethylcellulose; NHS-biotin, N-hydroxysuccinimide ester of biotin; NaP, sodium phosphate buffer (50:50 mono and dibasic sodium phosphate); Dm DNA or RNA, *Drosophila melanogaster* nucleic acids; EM, electron microscopy.

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incubated for 1 hr at room temperature. A total of 2 mg NaBH_4 /1 mg RNA was added in 4 portions over a period of 80 min. The solution was allowed to stand at room temperature for an additional 30 min and the NaBH_4 decomposed by addition of 0.1 - 0.2 ml of 4 M NaAc buffer (pH 5.0). The contents were dialyzed at 0°C against 0.01 M NaP buffer, pH 6.8. All of the steps up to and including reduction with NaBH_4 were performed in the dark.

For the spectrophotometric determination of concentration, we use molar extinction coefficients of 9.64×10^4 at 410 m μ , 5.4×10^5 and 8.3×10^5 at 260 m μ for cytochrome-c, 4S RNA, and 5S RNA, respectively.

Free cytochrome-c was removed from the reaction mixture by passage over a 3 x 1 cm column of carboxymethyl cellulose (CMC) that had been reequilibrated with 0.01 M NaP buffer. The CMC had been washed with acid and base according to the directions provided by the supplier. The sample was loaded and washed in 0.01 M NaP buffer. Free tRNA and the tRNA-cytochrome-c conjugate come through in the first wash.

Hydroxypatite (HAP, Bio-gel HTP from Bio-Rad) was hydrated by boiling for 10-20 min in 0.01 M NaP, pH 6.8, washed with 0.5 M NaP buffer, and re-equilibrated in 0.01 M NaP. For each washing the suspension was swirled gently and allowed to settle for 10-15 minutes before decanting the finer particles. The HAP was packed into a 2 x 0.5 cm column in 0.01 M NaP and the mixture of tRNA and tRNA-cytochrome from the CMC column applied. The column was successively washed with 10 ml volumes of 0.1 M, 0.15 M, 0.3 M and 0.5 M NaP buffer, pH 6.8. Fractions (0.5 - 1 ml) were collected, and assayed by spectrophotometry.

Addition of Biotin. The N-hydroxysuccinimidyl (NHS) ester of ^{14}C -biotin was prepared as described (1). The 1:1 conjugate, tRNA-cytochrome-c, from the HAP column was treated with an approximately 100 fold excess of NHS- ^{14}C -biotin under conditions previously described (3). Free biotin was removed by dialysis against 0.01 M NaP and the tRNA-cc-biotin stored at -20°C .

EM Labels. Ferritin-avidin was a gift from L. Angerer (1). Polymethylmethacrylate spheres (a gift from N.D. Hershey) were conjugated to avidin as previously described (3). One of the sphere-avidin preparations was a gift of M. Pellegrini.

Heteroduplex formation and electron microscopy. Heteroduplex formation between Dm plasmids containing 4S or 5S genes with DNA of the vector, Colicin E1, has been described (6,7).

$\phi 80\text{h}/\phi 80$ psu $_3$ heteroduplexes were formed as follows: a solution containing equal amounts of $\phi 80\text{h}$ and $\phi 80$ psu $_3$ bacteriophage was treated with 20 μl

of 0.2 M EDTA (pH 8.0) for 30 min on ice. Complete lysis of the virions and denaturation of DNA was accomplished by addition of 20 μ l of 1 N NaOH for 10-15 min at room temperature. The mixture was neutralized with 30 μ l of 2.5 M Tris HCl. tRNA-cc-biotin was added and the volume was made to 200 μ l with 3X recrystallized formamide (99%, Matheson, Coleman and Bell). The final DNA and tRNA concentrations were 3 μ g/ml and 10-30 μ g/ml. Hybridization was performed by dialysis of this mixture against 40% 3X recrystallized formamide, 0.1 M Tris, 0.3 M NaCl, 1 mM EDTA, pH 8.0 at 40°C for 40-50 min. Subsequent manipulations were as described (2). The concentration of spheres-avidin in labeling experiments was approximately 100 μ g/ml. No removal of excess spheres-avidin was attempted, as an effective procedure to do so is not available.

All electron microscopy and measurements of molecular lengths were done as previously described (2, 6, 7). Single and/or double stranded ϕ X 174 DNA (5370 nucleotides or nucleotide pairs, (11)), was used as a length standard.

Preparation of Dm 125 I 5S RNA. 125 I-5S RNA was prepared essentially according to Orosz and Wetmur (12). The reaction mixture contained, in the order of addition, the following: 10 μ l H₂O (double distilled), 3-5 μ l Dm 5S rRNA (1.9 mg/ml in 0.01 M NaAc, pH 4.8), 3 μ l of 1 M NaAc (pH 5.0), 10 μ l 125 I (Amersham, carrier free, 100 μ Ci/ml), and 3 μ l of freshly prepared TlCl₃ (ICN-K & K Laboratories, 18 mg/10 ml double distilled H₂O). The mixture was incubated at 60° for 20 min in a sealed siliconized 50 μ l pipette. The contents were transferred to 1 ml of 0.1 M NaAc buffer (pH 5.0) containing about 50-60 μ g of Dm 18S and 28S rRNA, and dialyzed against 0.5 M NaCl, 0.015 M NaH₂PO₄, 2 x 10⁻⁴ M EDTA, pH 6.0 at 0° (2 x 500 ml) and at 60°C (2 x 500 ml) and at 0°C again until no counts were detected in the dialysate. The 125 I-5S RNA preparation was then treated with 50 μ g/ml proteinase K (EM Laboratories, Inc.), phenol extracted, and further purified as described (4) or on Cs₂SO₄ gradients. Specific activities obtained in the different preparations ranged from 0.2 - 1 x 10⁸ cpm/ μ g.

Solution Hybridization of 125 I-5S RNA to DNA fractions. The contents of 5S genes in the several fractions of Dm DNA for the gene enrichment experiments were carried out by saturation hybridization using excess 125 I-5S RNA in solution. DNA solutions were denatured in 0.2 M NaOH, neutralized, and adjusted to the 80% formamide hybridization solution described in the next section. All of the samples contained 10⁻⁴ M KI in order to reduce background. Typical concentrations of DNA assayed in the respective fractions were 0.075 - 0.5 μ g/ml, 13-38 μ g/ml, and 26-400 μ g/ml, in the enriched, un-

fractionated, and depleted fractions, respectively. Reactions were carried out to a rot of 0.06 - 0.6 mol sec/liter. Samples were diluted 10 fold with 2 x SSC and treated at 37° for 1 hour with RNAase (100 µg/ml RNAase A, 4 units/ml T1 RNAase).

Gene Enrichment Procedure. RNA:DNA hybridization for the gene enrichment experiments was carried out in a high formamide solvent (13) which permits RNA:DNA hybridization but little or no DNA:DNA reassociation. Formamide was 3X recrystallized. DNA in 80% formamide, 2XSSC, was denatured by heating to 80°C for 10 min. A typical hybridization mixture contained 100 µg/ml Dm ³H-DNA, 10 µg/ml Dm 5S RNA-cc-biotin, 150 - 1000 µg/ml Dm 18 + 28S rRNA, all in 2XSSC, 80% formamide at 45°C for 30 min (rot = 6×10^{-2} mol sec l^{-1} for the 5S RNA). The sample was dialyzed at 0°C against 0.1 M NaCl, 1 mM Tris, 1 mM EDTA, pH 8.5, and passed over a Sepharose 2B column (19 x 1 cm) to remove excess 5S RNA-cc-biotin. Elution volume has previously been calibrated with Dm ³H-DNA and ¹²⁵I 5S RNA. The volume of the DNA fraction was reduced to about 100-500 µl by evaporation in a vacuum desiccator. In different experiments 50-150 µl of avidin-spheres (10-15 mg/ml) were added either during or after the evaporation. The solution was adjusted to 1 M NaCl and allowed to stand for 12-16 hours at room temperature or 48 hrs at 0°C. Spheres and DNA bound to spheres were separated from free DNA by banding in CsCl as described (4) except that centrifugation was performed for 48 hrs. The amounts of DNA in the different fractions were determined by ³H counting. DNA was released from the spheres and the RNA hydrolyzed by treatment with 0.2 M NaOH at 100°C for 20 min (14) or at 37°C for 16 hours. The 5S gene content of the several fractions was determined as described above.

RESULTS AND DISCUSSION

Preparation and Purification of RNA-cytochrome-c-biotin. tRNA or 5S RNA was reacted with cytochrome-c as described. The first step in the purification of the reaction product from the starting materials is passage over a CMC column in 0.01 M NaP buffer. Spectrophotometric monitoring showed that neither tRNA nor tRNA-cytochrome-c binds to the column whereas the free cytochrome-c does. The crucial step in the purification of RNA-cytochrome-c from unreacted RNA is HAP chromatography. As shown in fig. 1, tRNA elutes from HAP in a 0.15 M NaP wash, whereas RNA-cytochrome-c is eluted by 0.3 M NaP. The absorbance peaks at 410 and 260 mµ show that the material being eluted at 0.3 M NaP is the conjugate with 1:1 molar ratio.

Several comments should be made about the procedure. By using a 10-15 fold excess of cytochrome-c to RNA we decrease the probability of forming

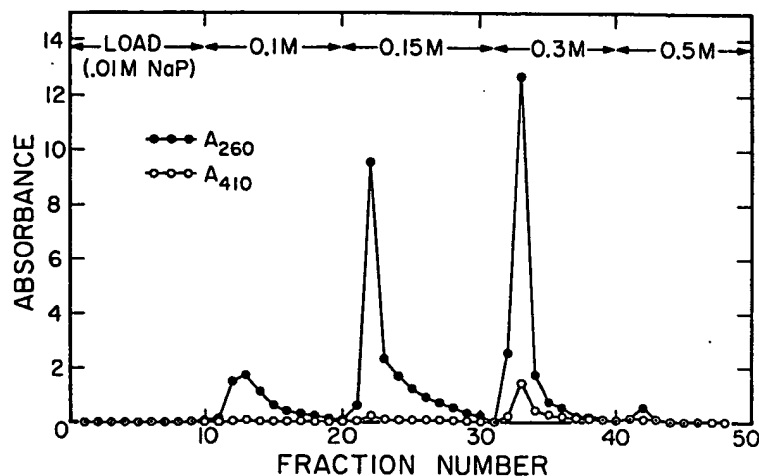


Fig. 1. Elution profile of tRNA-cytochrome-c from a HAP column. Batchwise elution of the reaction components was performed as described in Methods. tRNA and cytochrome-c concentrations were measured by absorbance at 260 m μ and 410 m μ , respectively. The column was loaded in 0.01 M NaP, pH 6.8. No A₂₆₀ or A₄₁₀ was observed in the washes with this solvent. The concentrations of tRNA and of cytochrome-c in the elution peaks were calculated as (tRNA) = (A₂₆₀/5.4 $\times 10^5$) mole liter⁻¹ and (cyt-c) = (A₄₁₀/9.6 $\times 10^4$) mole liter⁻¹. The total amounts of tRNA and cytochrome-c in the peak eluting at 0.3 M NaP above are 35 and 34 nmoles, respectively.

molecules such as (RNA)₂-cytochrome-c. The elution profiles vary somewhat with the batch of HAP used and with its preparation. Our results have been reasonably reproducible when the HAP is freshly prepared for each experiment as described in Materials and Methods. Nevertheless it is necessary to monitor the absorbance profiles for each new purification, and to make slight adjustments of the salt concentrations of the elution buffers accordingly.

The final results of all experiments on the preparation of RNA-cytochrome-c and of RNA-cc-biotin are given in table I. The yields of RNA-cytochrome-c in some of the earlier experiments were poor. We now suspect that the *Drosophila* tRNA used was charged with amino acids, but we failed to include a deacylation step, hence the poor yield in these experiments. The best yields with deacylated *E. coli* tRNA are approximately 50%. For 5S RNA it proved necessary to pretreat the cytochrome with iodoacetate to inactivate RNAase, otherwise the 5S RNA was extensively degraded. We believe that this step is advisable for tRNA preparations also, but it was not done in the experiments of table I. Heating the RNA sample to 80° before coupling appears to improve the yield, possibly because it causes dissociation of aggregates formed during lyophilization or ethanol precipitation.

Table I
Effect of Different Treatments on Yields of RNA-cytochrome-c-biotin

RNA	Treatment	% Yield (1:1 RNA/cyto-c)	Molar excess of biotin/RNA	Number of biotins/RNA-cyto-c
<i>E. coli</i> tRNA	no heat step	28.7	50	5
<i>E. coli</i> tRNA	no heat step	12.7	33	3
<i>E. coli</i> tRNA	80°C, 1 min	46	190	7
<i>E. coli</i> tRNA	80°C, 1 min	48	100	7
^a Dm tRNA	80°C, 1 min	11.2	240	9
Dm tRNA	80°C, 1 min	18.7	100	10
^b <i>E. coli</i> 5S rRNA	no heat step	30	100	7
<i>E. coli</i> 5S rRNA	70°C, 10 min	55	100	5
Dm 5SrRNA	70°C, 10 min	56	100	9
Dm 5S rRNA	70°C, 10 min	43	100	5

^aAs mentioned in the text, Dm tRNA was not deacylated and hence lower yields.

^bResults with 5S rRNA are those where cytochrome-c was pretreated with iodoacetate as described in Methods. Initially, when the iodoacetate step was omitted, the yields of the final product were low (0-5%).

As shown in table I, a molar excess of 50-100 fold of NHS-biotin to tRNA-cytochrome-c was used in order to obtain a final product with 3-10 biotins per cytochrome. Control experiments with unconjugated RNA gave undetectable binding of biotin after treatment with NHS-biotin and dialysis.

Cytochrome-c is positively charged and does not elute from the negatively charged resin, CMC, until the NaCl concentration is raised to approximately 5 M, whereas both tRNA and tRNA-cytochrome-c are negatively charged and do not bind to the column even in 0.01 M NaP. The crucial step in the purification is the HAP chromatography step. Unligated cytochrome-c elutes with approximately 0.5 M NaP, free tRNA with about 0.15 M NaP, and the 1 to 1 RNA-cytochrome conjugate at 0.3 M NaP. Several other separation methods were tried without success, including DEAE chromatography both in denaturing and nondenaturing conditions, CsCl centrifugation and gel filtration.

EM Mapping: The ϕ 80psu₃⁻/ ϕ 80 heteroduplex. This heteroduplex is a convenient test system for tRNA mapping techniques. As shown in previous studies (15, 16) and sketched in fig. 2, the heteroduplex loop consists of a 3100 nucleotide segment of *E. coli* DNA and a 2100 nucleotide single-strand

segment of $\phi 80$ DNA. The substitution begins at the att site of $\phi 80$ DNA. The *E. coli* single strand segment contains 1 tRNA^{tyr} gene at a position 1100 nucleotides from the att junction. Electron micrographs of two heteroduplexes labeled with spheres-avidin are shown in fig. 2. Micrographs (not shown) of heteroduplexes labeled with ferritin-avidin are comparable in appearance to those obtained by other methods (2, 15). A histogram of the spheres positions is given in Fig. 3. The results are in accordance with previous mapping data.

A considerable background of free spheres is evident in the micrograph. We have not found a procedure for separating unbound spheres from those attached to DNA, comparable to the sodium iothalamate buoyant banding procedure (1) that can be used to separate free ferritin from ferritin

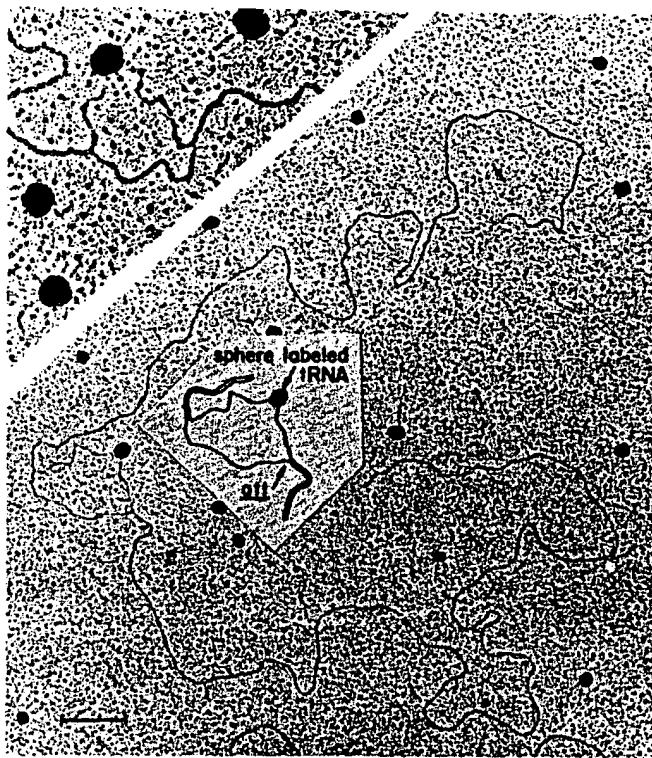


Fig. 2. Electron micrographs of sphere labeled tRNA genes on $\phi 80/\phi 80\psi 3^-$ heteroduplex. One complete molecule is shown, with an inset sketch of the heteroduplex loop. The att site and the fork at the other end of the substitution loop are 23.8 and 19.3 kb from the left and right ends of the heteroduplex, respectively (15); therefore they are readily distinguished. An inset photograph of the heteroduplex loop of a second molecule is shown; the magnification is 2X that of the other photo. Bar = 1 kb.

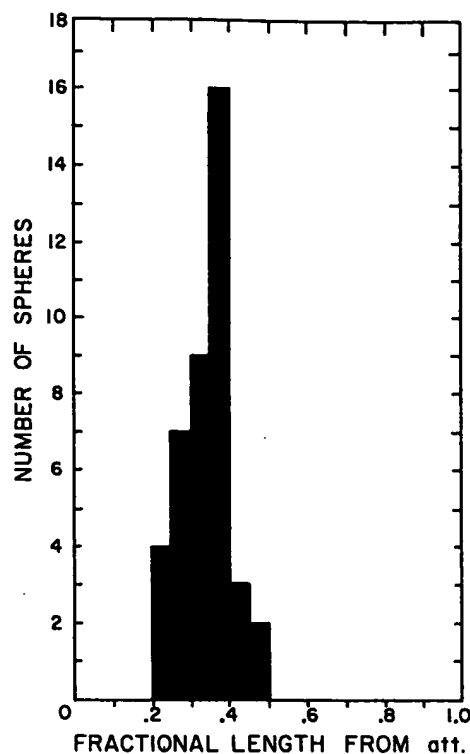


Fig. 3. Histogram of map position of tRNA^{tyr} gene on the single strand of bacterial DNA of the $\phi 80/\phi 80$ psu_3^- heteroduplex. The horizontal coordinate is the fractional distance from the att site to the label. The measured position of the label is 1100 (\pm 66) nucleotides from the att site.

labeled heteroduplexes. It may be noted however, that the spheres in the background seem to avoid the DNA strands.

By counting random fields we estimate labeling efficiencies per gene of 50-60% for both ferritin-avidin and the sphere-avidin labels using the cytochrome-c bridge. In experiments with the pentane diamine linker, the labeling efficiency with spheres was negligibly small (N.D. Hershey, personal communication). Presumably the greater length of the cytochrome-c linker is responsible for the efficient labeling in the present method. Nevertheless it should be noted that the efficiency of labeling either with spheres-avidin or ferritin-avidin is at best about 60%. The reason why a figure closer to a 100% is

not achieved is not known.

EM Mapping. Drosophila 4S and 5S genes. Studies from this laboratory on the mapping of 4S RNA genes on a 9.3 kb segment of *Drosophila melanogaster* (Dm) DNA contained on the recombinant DNA plasmid pCIT12 have been reported (7). The mapping data were obtained by EM mapping, using the technique described in detail in the present paper with a ferritin-avidin label, and by restriction endonuclease - hybridization mapping.

Electron micrographs from additional mapping experiments with the avidin-sphere label on the plasmid pCIT12 are shown in fig. 4. A histogram of the observed positions of the tRNA genes on the Dm insert is shown in fig. 5. In the previous study as well as in the present one labeled sites (genes) were found at the 3 positions 1.38 ± 11 , 4.59 ± 0.17 and 8.38 ± 0.26 kb from the defined left end of the Dm insert. An additional gene mapping in the position 5.6 - 6.2 kb was found by restriction endonuclease mapping but was not detected at an appreciable frequency in the ferritin-avidin studies. The histogram in fig. 5 shows that this gene, mapped at 5.89 ± 0.36 kb was labeled at a frequency lower than that for the other genes but at a clearly detectable level in the present sphere-avidin studies. We do not know at present whether this improved labeling efficiency is due to the fact that the avidin spheres contain more avidins per label (8-10) than do the ferritin labels (1-2), or due to some other unknown factor.

Ferritin-avidin mapping studies, using the present techniques, on the recombinant plasmid pCIT9 with an insert carrying 3 Dm 5S RNA genes have been reported (6). *Drosophila* 5S genes are tandemly repeated with a regular spacing of about 380 nucleotides. Our labels -- ferritin-avidin or spheres-avidin-- are multivalent, in that they contain several biotin binding sites per label. Many tangled structures were seen because one label was attached to several hybridized RNA-biotin molecules along a DNA strand. Such molecules cannot be accurately analyzed; nevertheless we have estimated that the overall efficiency of labeling per gene is 40-50% in the various experiments.

As an overall evaluation then, the present method gives 40-60% labeling efficiency in practical problems. Resolution is probably limited by the diameters of the labels - about 200 Å for ferritin-avidin and 600 Å for spheres-avidin. At present, difficulties are encountered with closely spaced genes because of the multivalent character of the labels. Further work is needed to develop a ferritin-avidin conjugate with only one avidin per ferritin and with a high efficiency of labeling, so that closely spaced multiple genes can be mapped.

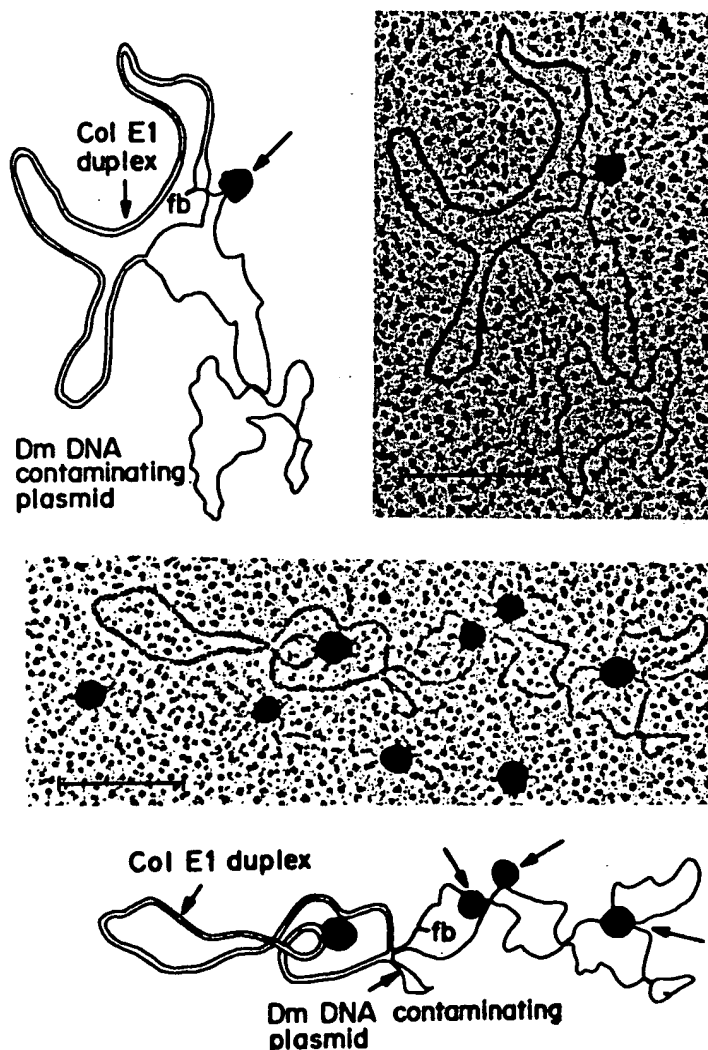


Fig. 4. Electron micrographs of sphere labeled tRNA genes on a single strand of the recombinant plasmid pCIT12. For a full explanation of the structures, see (7). Briefly, the molecules shown are heteroduplexes of pCIT12, which is ColE1 fused to a 9 kb Dm insert, with a second contaminant plasmid consisting of ColE1 fused to another short Dm insert. A secondary structure feature, fb, on the 9 kb single strand serves to orient that strand. The single tRNA gene labeled in the upper micrograph is at the position 8.38 ± 0.26 in fig. 5; the three sphere labels in the lower micrograph are at the positions 1.38 ± 0.11 , 5.80 ± 0.36 and 8.38 ± 0.26 in fig. 5. In addition there is a sphere nonspecifically attached to the ColE1 duplex. These are rare. Bars = 1 kb.

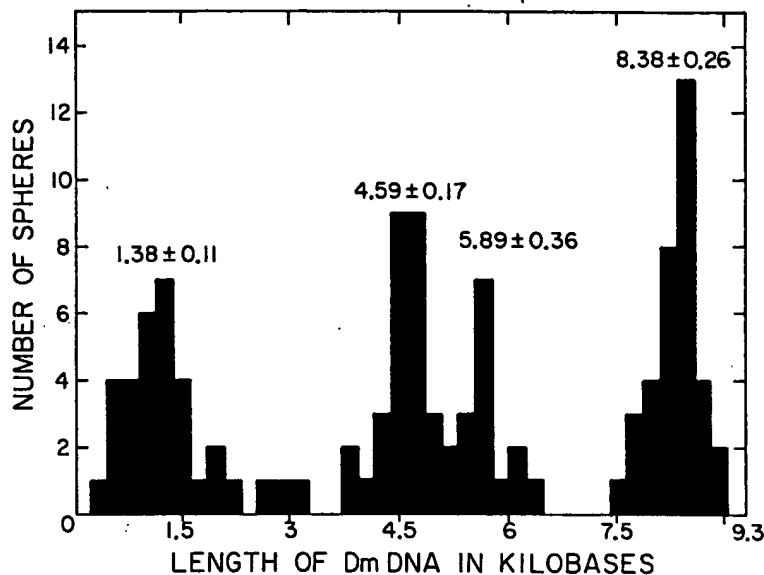


Fig. 5. Histogram of the distribution of Dm tRNA gene positions on the pCIT12 plasmid. The measurements were done as described (7). In addition to the 3 genes found in the previous ferritin mapping study, a 4th gene at 5.89 kb was labeled here, although to a lesser extent. Its position is in agreement with that determined for the 4th gene by the restriction endonuclease mapping.

Gene Enrichment. In these experiments, high molecular weight *Drosophila* DNA is incubated with 5S RNA-cc-biotin. Hybridization is carried out in a high formamide solvent under conditions where RNA:DNA association is favored over DNA:DNA association (13). Those DNA strands which hybridize to the 5S RNA are separated from all other strands by reaction with avidin-spheres and buoyant banding. These experiments are basically identical to those previously described for the enrichment of the rRNA genes of *Drosophila* (4), except that, as appropriate for the short length of the 5S RNA, we use a terminal cytochrome-c bridge between the RNA and the biotin instead of random crosslinking of cytochrome-biotin to the RNA by CH_2O .

There are 160 5S RNA genes of *Drosophila* (17). They are tandemly repeated with a repeat spacing of about 380 nucleotides. They may occur as two clusters of approximately 80 genes each or there may be a single cluster of length 63.4 kb containing all of the genes, as suggested by restriction digest studies of the chromosomal DNA (17, 18). All told, the genes plus spacers constitute 1.73×10^{-4} of the haploid Dm genome (1.8×10^8 base

pairs). In the present experiments the bulk of the single strands of Dm DNA ranged in length from 10-100 kb. Thus the strands carrying 5S genes would have 25 or more 5S RNA-cc-biotin molecules hybridized. (There is every theoretical reason to expect that the efficiency of RNA:DNA hybridization is almost 100%.) For a strand of length 10 kb with one sphere attached the weight fraction of sphere mass is 0.96. If several spheres are attached per 10 kb the weight fraction is still closer to 1. Thus by the rules formulated by Pellegrini et al. (5) the buoyant density of the DNA sphere complex would be close to that, 1.25 g/ml, of uncomplexed spheres. The efficiency of labeling in the electron microscope experiments was 50% per gene. Just how many avidin-spheres would be bound to a 10 kb strand carrying 25 hybridized 5S RNA-cc-biotin molecules is uncertain because we do not know how many of these biotins would bind to a single avidin-sphere rather than to different avidin-spheres.

The results of several gene enrichment experiments are presented in table II. In all three experiments, almost all (greater than 88%) of the 5S genes were recovered in the enriched fraction. In the first experiment, about 1.5×10^{-2} of the total DNA was in the enriched fraction whereas in the second and third experiments only about 6.5×10^{-4} of the DNA was in the

Table II

Results of Enrichment for 5S rDNA from Total Drosophila DNA

	% Total DNA in		% 5S rDNA in		Enrichment Factor
	Enriched	Depleted	Enriched	Depleted	
Theoretical Experiment	0.0173	99.923	100	0	5780
1	1.48	98.52	99.9	not detected	67
2	0.067	99.933	91.5	8.5	1351
3	0.063	99.937	88.8	9.8	1411

The volume of the DNA after the Sepharose 2B step is usually 7-10 ml. The salt concentration is 0.1M. This volume is reduced by evaporation by a factor of 10 and the salt concentration raised to 1.0 M for sphere labeling under conditions of minimal electrostatic interaction between the positive avidins on the spheres and the negative DNA. In the first experiment, the spheres were added before the evaporation step. Perhaps this caused more quasi-irreversible electrostatic binding between spheres and DNA. In experiments 2 and 3, spheres were added after evaporation. In the 3rd experiment, labeling was done at 0° for 48 hr instead of at room temperature for 16-24 hours.

enriched fraction. Therefore in the first experiment the 5S DNA after one cycle of enrichment was about 1% pure, whereas in the second and third experiments it was about 25% pure. In control experiments where no RNA was added approximately 3.4×10^{-4} of the DNA was found in the sphere band. Therefore of the total of about 6.5×10^{-4} DNA in experiments 2 and 3, about 50% may be attributed to general binding or trapping of DNA by the spheres, 25% to specific gene enrichment, and 25% to other causes, possibly formation of networks or partially duplex DNA with a strand bearing a 5S gene (see below).

The two major technical differences between experiment 1 and the more successful experiments 2 and 3 were:

- 1) In the latter experiments, the excess of unlabeled (no cytochrome-biotin) rRNA (which included 5.8S rRNA as well as 18 and 26S) added was 100 times the amount of 5S RNA, whereas in the first experiment there was only a 15 fold excess. Whereas 5S genes plus spacers make up 1.7×10^{-4} of Dm DNA, rRNA genes plus spacers constitute 6.4×10^{-3} ; therefore it is necessary to completely compete out hybridization of any biotin labeled rRNA fragments present as contaminants in the 5S preparation with the cold rRNA. In their filter hybridization experiments, Tartof and Perry (19) found that it was necessary to use a 100 fold excess of unlabeled rRNA in order to accurately assay for the number of 5S RNA genes.

- 2) As explained in a footnote to table II, there was a difference in a concentration step of the sphere-avidin-DNA mixture between experiments 1 and experiments 2 and 3 which may have decreased the amount of non-specific binding in the latter.

A small fraction of the sphere band from the CsCl gradient was directly diluted into formamide solution and spread for electron microscopy. The DNA structures observed were of the following types: a) Single strands with one or many spheres. Strands with many spheres were tangled and condensed around the spheres, as expected in view of the close spacing of the genes and the several avidins attached to each sphere. b) Single strands with no spheres attached. These were presumably released from the spheres by breakage. c) Molecules that were partially duplex, due to some DNA:DNA reassociation. d) Networks of single strands, perhaps due to tangling of the long strands in the high salt medium. Factors (c) and (d) may contribute to the amount of non-coding strands in the enriched fraction. The DNA strands observed had about the same length distribution as the input DNA, showing that the gene enrichment procedure does not cause much chain breakage.

Further discussion. In several test systems, the efficiency of labeling with the cytochrome-c-biotin attached to the 3' terminus of 4S and 5S RNA was 50% with either avidin-spheres or ferritin-avidin. The efficiency of labeling by spheres-avidin for cytochrome-biotin randomly crosslinked to RNA with CH_2O is reported to be substantially lower than this figure (4). The present method could be applied to genes for long RNA's as well as for short ones. For long RNA, it would be advantageous to degrade the RNA to a length of 100-400 nucleotides, and expose new 2', 3' OH ends with alkaline phosphatase before coupling to cytochrome-c. Thus one would provide several cytochrome-biotin affinity labels per gene. In general then, the present technique appears to be a very useful addition to methods of gene enrichment and electron microscope gene mapping.

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